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TITLE: Bone Marrow Microenvironmental Control of Prostate
Cancer Skeletal Localization

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14. ABSTRACT The purpose of this study is to analyze the role of prostate cancer derived PTHrP and its ability to impact the bone marrow microenvironment of the skeletal metastasis. During the first year of the project, PC-3 cell lines expressing luciferase and with altered PTHrP levels were generated and their activity verified in <i>in vitro</i> and <i>in vivo</i> assays. The PC-3 cell lines with lowered PTHrP levels (via shRNA knock down) developed smaller tumors. The PC-3 cell lines with lowered PTHrP levels showed a trend toward lowered hematopoietic progenitors in the bone marrow. Altering the bone marrow microenvironment with an agent that reduces HPCs (cyclophosphamide) increased tumor engraftment. Altering the bone marrow microenvironment with zoledronic acid (a proven anti-tumor therapy) increased HPCs. These studies have already provided early data that suggest there are key and controllable events that can be triggered in the bone marrow which are conducive to tumor growth.				
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INTRODUCTION

Prostate carcinoma metastasizes to skeletal sites where bone remodeling is active and engages the bone marrow niche in an unstable cascade with dysregulated bone resorption and formation. Numerous factors in the bone marrow niche have been implicated that support tumor growth. This proposal is focused on parathyroid hormone related protein (PTHrP), a tumor-derived factor that increases angiogenesis and enriches the bone marrow complement of hematopoietic stem cells (HSCs) and hematopoietic progenitor cells (HPCs). The overall hypothesis is that: Prostate cancer-derived PTHrP increases hematopoietic cells which in turn support tumor localization and growth in the bone marrow microenvironment. Two specific aims will validate this hypothesis using novel but well characterized animal models accompanied by *in vitro* cell biologic approaches. The first aim will determine the contribution of prostate cancer-derived PTHrP acting as a stem cell factor to facilitate prostate cancer residency in the bone microenvironment. Aim two will identify a pro-angiogenic impact of prostate cancer-derived PTHrP and elucidate its role in prostate carcinoma residency in the bone microenvironment. Prostate cancer lines expressing varying levels of PTHrP will be used in models where angiogenesis is measured and then altered to verify the PTHrP angiogenic response and dependence for the tumor impact on hematopoietic cells and the osteoblastic response.

BODY

The first year of this project started with recruiting and hiring a post-doctoral fellow. Dr. Serk-In Park, a recent PhD graduate from M.D. Anderson was hired and acclimated to the laboratory and procedures. He has been a fantastic addition to the project and is now 'up to speed' and making noted progress on the aims. We discovered problems with our PC-3 PTHrP knock down cells as they were losing their phenotype when implanted *in vivo* and some lots were contaminated with mycoplasma. Hence new cell lines were generated and carefully analyzed to verify the phenotype and characterize their *in vitro* and *in vivo* behavior.

Task 1: Aim 1, Strategy 1: Impact of PCa-derived PTHrP on hematopoietic cells (HCs)

To investigate the role of prostate cancer derived-PTHrP on bone marrow microenvironment, we developed prostate cancer cells with reduced PTHrP expression level. Luciferase-labeled PC-3 cells were used for this aim. Short-hairpin RNA sequence targeting PTHrP (NM_198966) were designed (GG GCA GAT ACC TAA CTC AGG A) and double strand oligonucleotide were synthesized (Invitrogen) and inserted into the lentiviral expression plasmid (pLenti4/Block-iT™-DEST destination vector, Block-iT™Inducible H1 Lentiviral RNAi system from Invitrogen). Lenti-viral vectors were produced by 293T packaging cells (University of Michigan Virus Core). PC-3^{Luc} cells (35mm dish, in duplicate) were transduced with 1ml of viral supernatant in the presence of 6µg/ml polybrene. After two days, cells were transferred to 10cm dish and grown in selection media (200µg/ml Zeocin, Invitrogen). After two-weeks in selection media, clones were selected with clonal selection rings. Cells were then expanded to 10cm dish and frozen for stock.

To measure PTHrP expression level, immunoradiometric assay were performed (Figure 1). One million cells were plated on 6-well plates in complete RPMI-1640 media. After 24 hours, cells were washed with PBS once and added 1% FBS RPMI-1640 media (2ml) in duplicate. After 48 hours, supernatant media were collected and added protease inhibitor cocktail. Supernatants were then analyzed with an IRMA assay (Diagnostic Lab.).

Using these cell lines we first evaluated their proliferative potential *in vitro*. Cells were plated and cell numbers evaluated over time (Figure 2). There was a trend toward the highest PTHrP producers having higher cell numbers over time, but this was not significant. Next we performed a subcutaneous implant model in order to verify that the cells maintain their PTHrP expression *in vivo* (Figure 4). Three lines were selected. The empty vector control has the highest PTHrP expression, and a medium and low expressing PTHrP. Tumors were monitored by bioluminescence and tumor size evaluated at sacrifice. There was a statistically significant increase in tumor size that mirrored the PTHrP expression levels with higher PTHrP and larger tumor growth.

The subcutaneous tumor study was also utilized to map the HPC and EPC cells in the bone microenvironment. This has been performed once to date and the data suggests a trend (Figure 4) toward higher PTHrP expressing tumors having greater numbers of HPCs. We are currently repeating this experiment with greater n values. Endothelial progenitor cell markers are also under investigation in this project and we have been optimizing the markers and conditions for this assay. Figure 4 shows the EPC values that suggest a trend; however, we are not yet comfortable with the markers as in separate experiments using the positive control of VEGF we do not see an increase in EPCs (data not shown). We will continue to optimize these conditions.

Cardiac inoculation experiments are underway. While we were developing the altered PTHrP PC-3 we were also optimizing the cardiac inoculation bioluminescent read outs with HPCs in the marrow. Two approaches were considered: cardiac inoculation with harvest of HPCs at a fixed time point (3 weeks after inoculation) versus harvest at a designated tumor size (via bioluminescence). The later appears to be the best approach. We are currently performing the cardiac inoculation with altered PTHrP expressing PC-3 cells.

Task 2: Aim 1, Strategy 2: Altered hematopoietic microenvironment and tumor localization

Progress: We have taken two approaches to alter the bone marrow microenvironment. The first is cyclophosphamide (CTX) treatment. Four to five weeks old male C57BL/6J mice and cyclophosphamide, a DNA-alkylating chemotherapeutic agent were used (Figure 5). Mice were pre-treated with a subclinical dosage of cyclophosphamide (350mg/kg) or equal amount of saline as control. One of our preliminary results of *in vivo* experimental PCa metastasis demonstrated that cyclophosphamide pre-treatment increased metastatic PCa tumor growth in the bone. Closer observation of the data suggests that increase of tumor growth may be a result of more tumor cells engrafted in the bone marrow space. This led us to hypothesize that cyclophosphamide pre-treatment increased permeability of the BM sinusoidal endothelium, leading to increased tumor cell extravasation. However, the increased tumor growth may also be due to bone marrow suppression (as observed in depletion of LSK cells in the cyclophosphamide pre-treatment group; Figure 6) or ablation of immune response (secondary to the BM suppression). To directly determine whether BM suppression is the primary reason of tumor growth secondary to the cyclophosphamide pre-treatment, PC-3^{Luc} tumor cells were directly injected into the BM space (tibia) after cyclophosphamide pre-treatment. Tumor growth was observed for 4-6 weeks via bioluminescence imaging (Figure 7). Tumor growth was also increased in the direct intra-tibial injection model which suggests that the CTX effect is not via initial seeding. This is in agreement with our original hypothesis in the proposal.

The second approach was to alter the bone marrow microenvironment with the administration of a potent bisphosphonate (Zoledronic acid). Interestingly, the HPCs were increased in the bone marrow with Zoledronic acid (ZA) administration (Figure 8). More stringent assays were performed for HSCs (using the SLAM family cell markers) and these were also elevated (Figure 9). Finally a definitive reconstitution assay was performed where cells were isolated from the marrow of ZA or control mice and inoculated into lethally irradiated mice. The numbers of mature T and B cells were measured over time (Figure 10). There was a significant increase in donor-derived T and B cells from mice treated with ZA. This indicates that ZA increases the HSCs in the microenvironment. As ZA inhibits tumor development in bone, this is consistent with our overall hypothesis.

Task 4: Aim 2, Strategy 1: PCa-derived PTHrP and angiogenesis via EPCs

See data described above and in Figure 4.

KEY RESEARCH ACCOMPLISHMENTS;

- PC-3 cell lines expressing luciferase and with altered PTHrP levels have been generated and their activity verified in *in vitro* and *in vivo* assays
- PC-3 cell lines with lowered PTHrP levels (via shRNA knock down) develop smaller tumors

- PC-3 cell lines with lowered PTHrP levels display a trend toward lowered hematopoietic progenitors in the bone marrow
- Altering the bone marrow microenvironment with an agent that reduces HPCs increases tumor engraftment
- Altering the bone marrow microenvironment with zoledronic acid (a proven anti-tumor therapy) increases HPCs

REPORTABLE OUTCOMES:

Park SI, Liao J, Li X, Berry J, McCauley LK. Chemotherapy-Induced Alterations of the Bone Marrow Microenvironment Contribute to Prostate Cancer Skeletal Metastasis. Submitted to the: The IX International Meeting on Cancer-Induced Bone Disease, October 28-31, 2009.

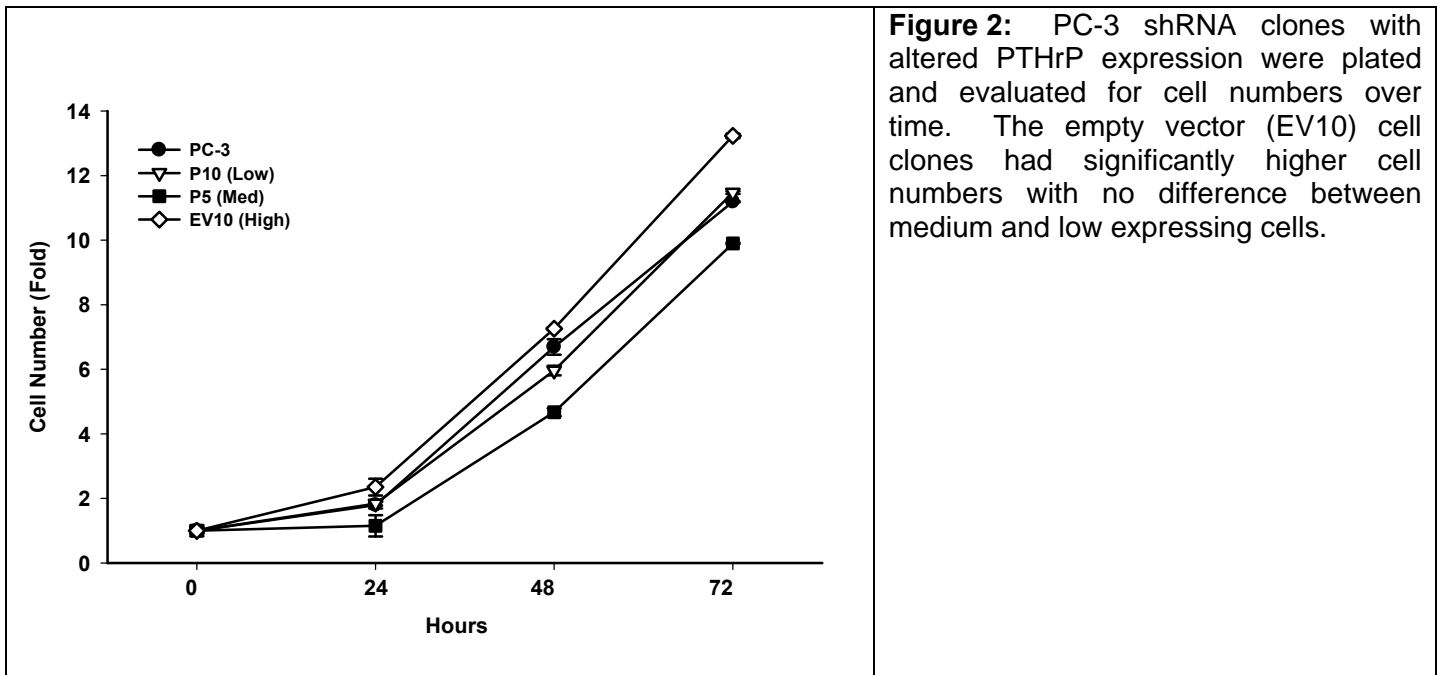
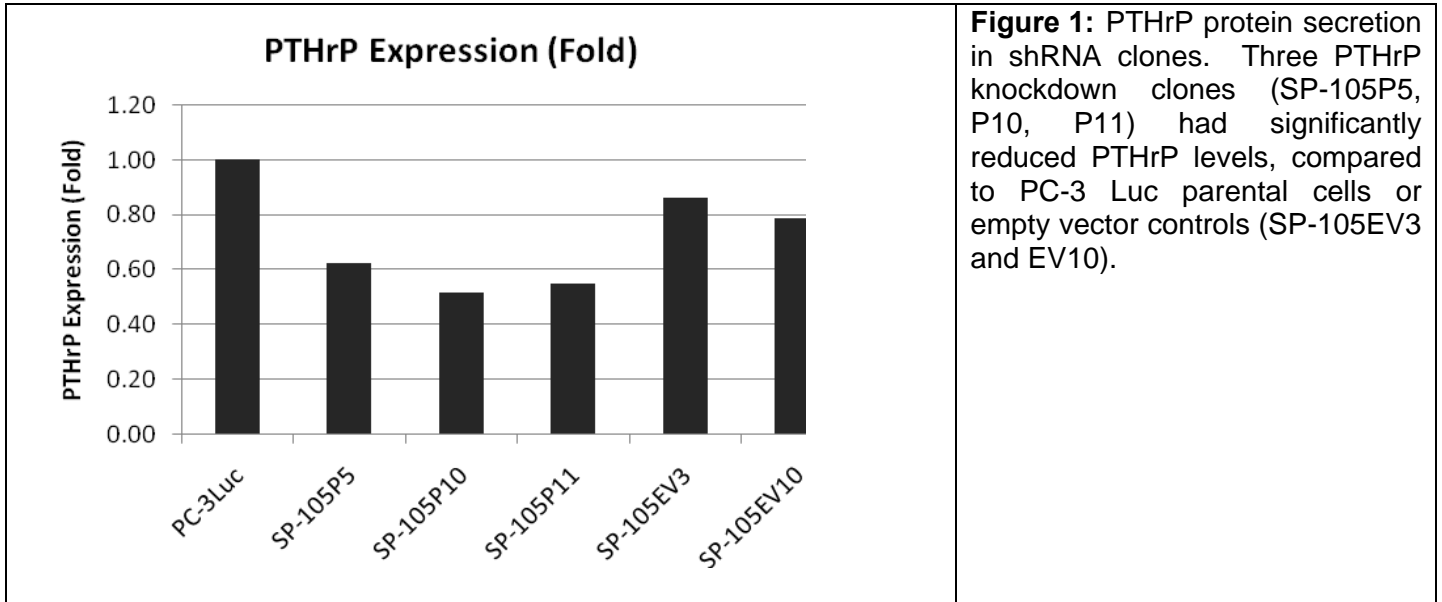
Li X, Park S, Liao J, Berry J, Pienta K, McCauley LK. Bone Marrow Calcium Levels Correlate with Bone Marrow Hematopoietic Progenitor Cells. Accepted for Oral Presentation and a Young Investigator Award at the American Society for Bone and Mineral Research meeting. September, 2009.

CONCLUSION: The first year of this project was a successful initiation of studies that will lead to new information about the tumor microenvironment and prostate cancer skeletal localization. New cell lines/model systems were generated and new data implicating PTHrP as an important mediator in prostate cancer was generated. Exciting data of altered bone marrow microenvironments and their ability to alter tumor localization and growth were discovered. Importantly, the implications of this early work suggest that there are key events that can be triggered in the bone marrow which are conducive to tumor growth. Further validation of this will likely lead to changes in the ways patients with early cancer diagnoses are treated.

REFERENCES: N/A

APPENDICES: two abstracts listed above

SUPPORTING DATA



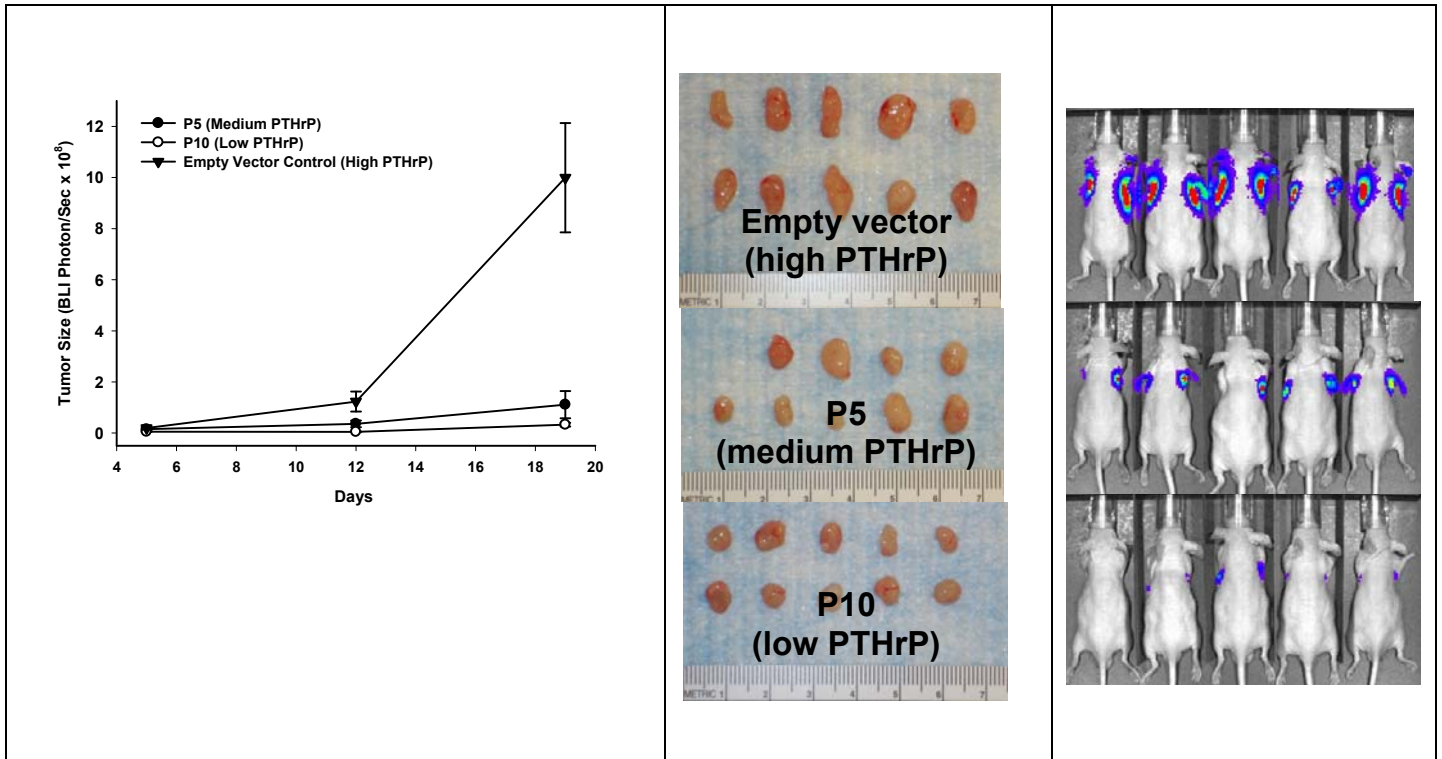


Figure 3: PC-3 shRNA clones with altered PTHrP expression were implanted subcutaneously and bioluminescence (as a reflection of tumor growth) monitored over time (plot on left, images on right). At sacrifice tumors were isolated (middle) and RNA harvested to verify that PTHrP levels were still altered as constructed (data not shown). Tumor size was directly related to PTHrP expression suggesting PTHrP is a tumor promoting factor.

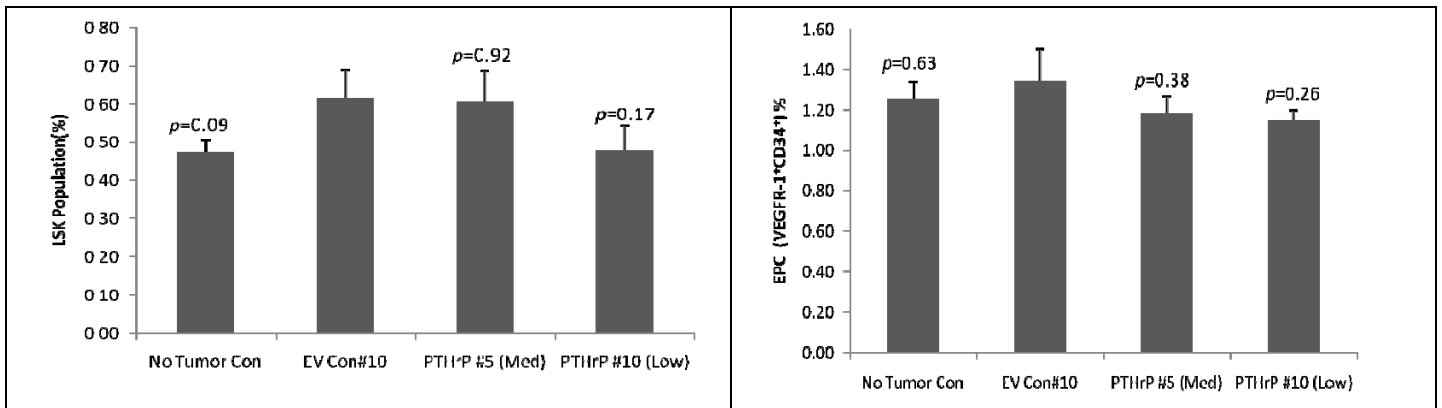


Figure 4: Flow cytometric analysis of HPCs (LSK cells) (left) or endothelial progenitor cells (EPCs) (right) in bone marrow from mice with subcutaneous tumors expressing altered levels of PTHrP. There was a trend towards increased LSKs with tumors and lower levels with lower PTHrP. These experiments are currently being repeated.

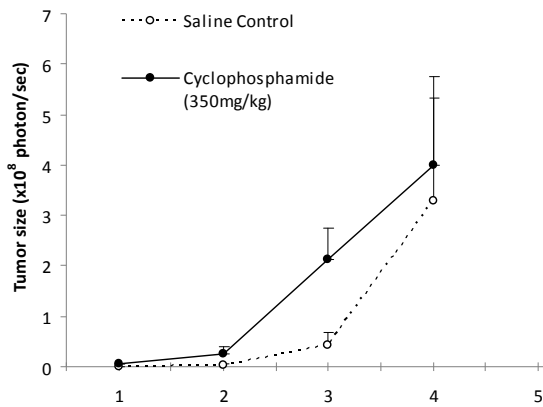


Figure 5 : Mice were pre-treated with cyclophosphamide (CTX) or vehicle (saline) control one time one week prior to cardiac inoculation of PC-3 cells and tumor growth monitored over time (weeks) by bioluminescence. There was a significant increase in tumor engraftment in cyclophosphamide-treated mice at three weeks.

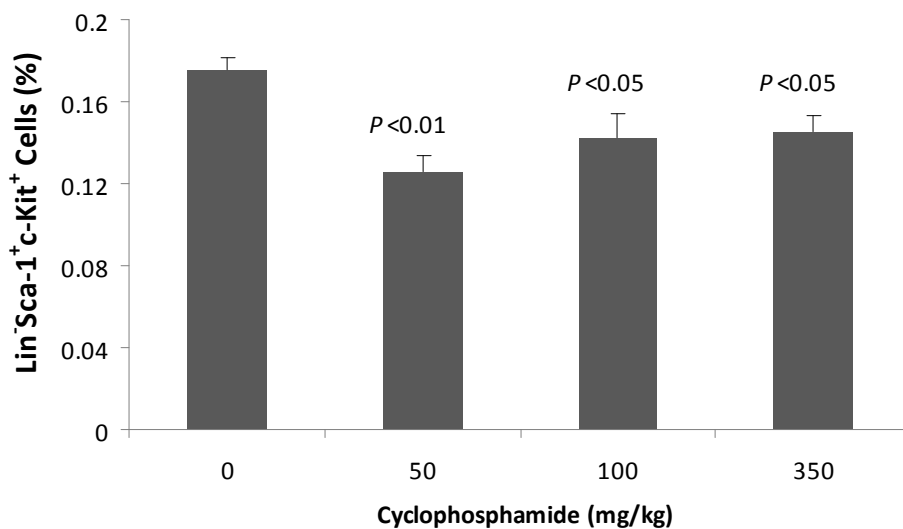


Figure 6 : Cyclophosphamide pre-treatment reduces HPCs (LSK) cells in the bone marrow microenvironment. Mice were pre-treated with indicated single dose of cyclophosphamide and one week later flow cytometry performed to analyze LSK cells.

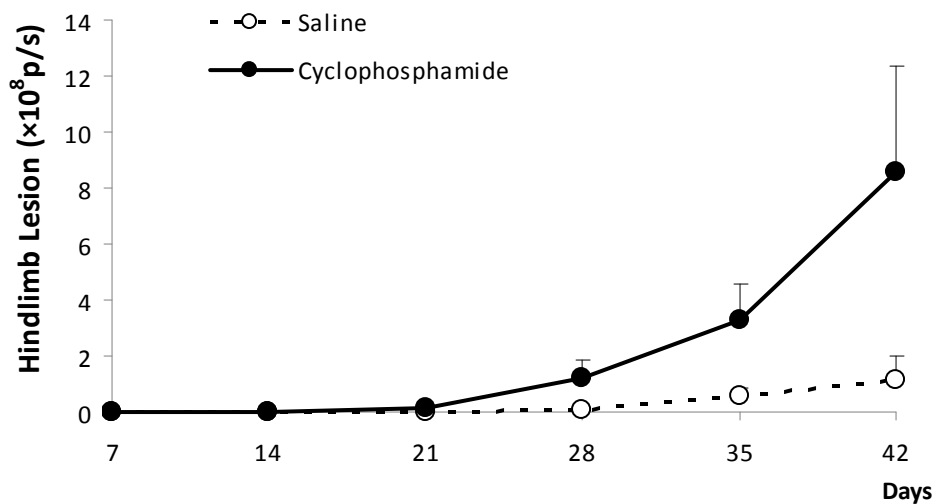


Figure 7 : Mice were pre-treated with cyclophosphamide (350 mg/kg) one week before intratibial inoculation of PC-3 cells into the tibia. Mice were followed by bioluminescence for 42 days after tumor inoculation. There was significantly greater tumor growth in cyclophosphamide treated mice.

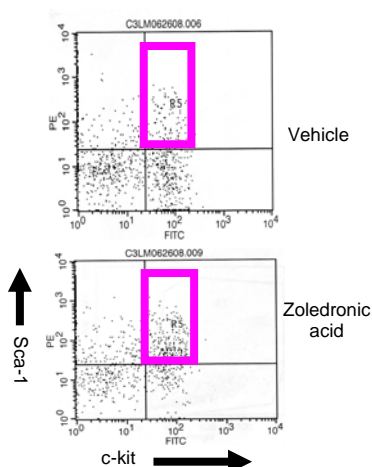
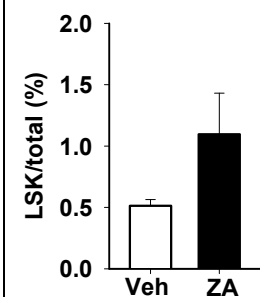
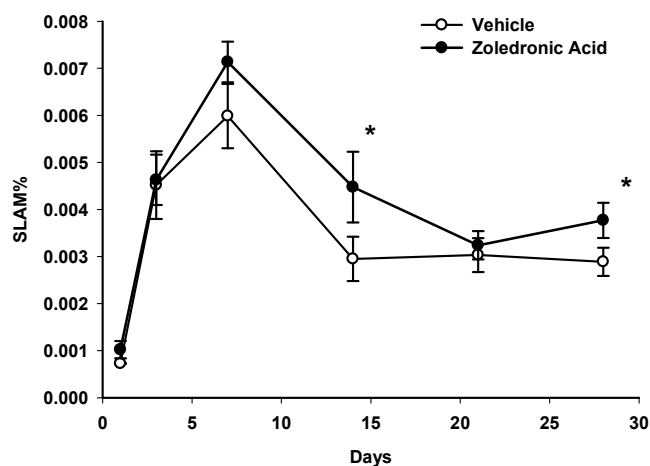


Figure 8: Mice were treated with Zoledronic Acid or vehicle control (5µg/2x/week) for 4 weeks. Bone marrow was harvested and flow cytometry performed for HPCs (lin-sca-1+c-kit+ or LSK cells). Zoledronic acid significantly increased the percentage of LSK cells in the bone marrow.



*p=0.05 vs. vehicle

Figure 9 : Mice were treated with Zoledronic Acid (5µg/2x/week) for 28 days. Mice were sacrificed at indicated time points and analysis of HSCs using the stringent SLAM family markers was performed. There was a statistically significant increase in HSCs at day 14 and 28 in zoledronic acid treated mice.

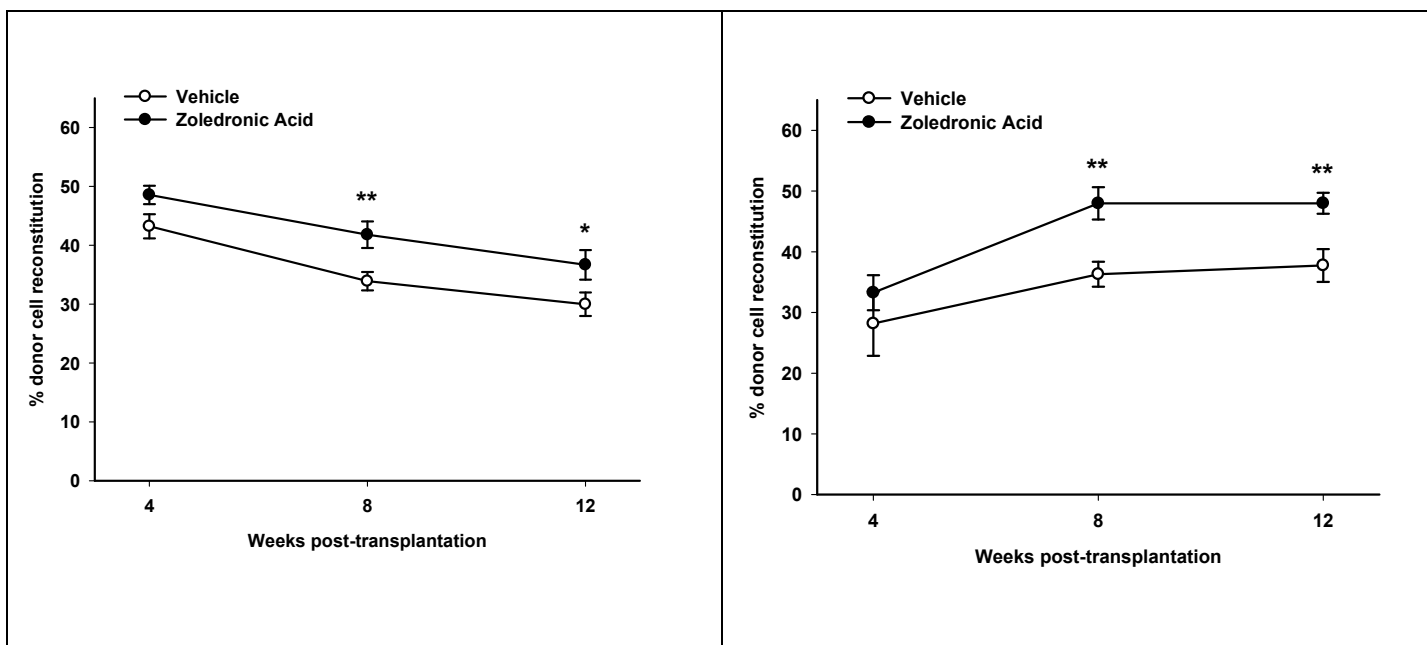


Figure 10: The long term reconstitution assay was performed by transferring bone marrow cells from zoledronic acid treated mice into lethally irradiated donor mice of a different strain (45.1 vs. 45.2). Peripheral blood was collected at various time points after transplantation and B-cells (B220 marker; left panel) and T-cells (CD3 marker; right panel) were evaluated. There was a significant increase in B and T-cells reflecting the increase in HSCs in Zoledronic Acid treated mice.

The IX International Meeting on Cancer-Induced Bone Disease

Title: Chemotherapy-Induced Alterations of the Bone Marrow Microenvironment Contribute to Prostate Cancer Skeletal Metastasis

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Abstract (750 words limit):

Bone is the preferred site of prostate cancer metastasis, and advanced-stage patients commonly develop metastatic bone lesions. Bone is a unique metastatic microenvironment, not only because of the calcified extracellular matrix, but also because of multiple types of constituting cells, including bone cells, hematopoietic cells, stem cells, stromal cells and endothelial cells. Tumor metastasis is a tightly regulated multi-step process, and metastatic growth of tumor cells in the distant organ is determined by highly specific interactions between tumor cells and the microenvironment. Indeed, as prostate cancer has long been known to home typically to bones enriched with red marrow, cells in the bone marrow (such as hematopoietic stem/progenitor cells) and stromal cells (such as bone marrow sinusoidal endothelial cells) likely play important roles in tumor localization and/or metastatic growth in the bone. In this study, the impact of alterations in the bone marrow microenvironment induced by a chemotherapeutic agent (cyclophosphamide) on prostate cancer skeletal metastasis was investigated.

Male athymic mice were pre-treated with a bone marrow suppressive chemotherapeutic agent (cyclophosphamide, 350mg/kg, i.p.) or saline (control) once ($n=15/\text{group}$). After 7 days, alterations in the hematopoietic cells were verified by flow cytometry in a subset of mice, and luciferase-labeled PC-3 prostate cancer cells (PC-3^{Luc}) were inoculated into the left cardiac ventricle of remaining test mice. Tumor cell localization and growth in the mandible and in the hind limb long bones were subsequently monitored via weekly bioluminescence imaging for six weeks. A single pretreatment with cyclophosphamide significantly reduced the hematopoietic stem/progenitor cell populations, as determined by flow cytometric analysis of Lineage⁻Sca-1⁺c-Kit⁺ (LSK) cells in the hind limb bone marrow. Interestingly, the bone marrow-suppressed mice developed significantly larger tumors in both hind limbs and mandible versus saline-treated mice. The tumor size difference was apparent as early as the week-2 time point. In addition, metastatic tumor incidence in the hind limb was significantly higher in the cyclophosphamide pre-treatment group at earlier time points (week-1, -2 and -3), compared to the saline-treated control group, suggesting that the increased skeletal metastasis was a result of increased tumor cell seeding. To test whether cyclophosphamide pretreatment disrupted bone marrow vascular barrier function leading to increased tumor cell extravasation, male C57B6J mice were pre-treated with cyclophosphamide (350mg/kg, i.p.) or saline ($n=13$, each). After 7 days, mice were perfused with Linger's lactate solution supplemented with heparin to completely flush blood, followed by perfusion with 3.7%

buffered-formaldehyde for fixation. Subsequently, mice were perfused with radiopaque monomeric compound (Microfil®) in liquid phase. After polymerization at 4°C overnight, femurs were dissected, decalcified and scanned with micro-CT (computed tomography) for visualization of polymerized vascular structures. This novel technique visualized very fine vascular structures (less than few μm in size, in the growth plates) in three-dimension. The micro-anatomic structure of the cyclophosphamide pretreated bone marrow sinusoids was significantly disrupted (i.e. abnormal constriction and enlargement of central sinusoids; discontinuity and tortuousness), compared to the saline treated control bone marrow. Lastly, to investigate the underlying mechanism of vascular changes in response to cyclophosphamide pre-treatment, the expression of angiogenic molecules in the bone marrow microenvironment was determined in response to cyclophosphamide in mice. Male athymic mice were pre-treated with cyclophosphamide or saline once ($n=5/\text{group}$), and bone marrow cells and serum were collected after 7 days. Quantitative RT-PCR (reverse transcription-polymerase chain reaction) results demonstrated increased protein levels of monocyte chemoattractant protein-1 (MCP-1, also known as CCL-2) and interleukin-6 (IL-6), both of which have angiogenic functions. In addition, serum VEGF (vascular endothelial growth factor) levels were increased in cyclophosphamide pretreated mice, as determined by ELISA (enzyme-linked immunosorbent assay).

In conclusion, chemotherapy-induced bone marrow suppression significantly increased experimental prostate cancer skeletal metastasis *in vivo*. In addition, a single dose treatment of cyclophosphamide disrupted bone marrow sinusoid vascular structures, suggesting the increased tumor metastasis was due to disrupted vascular barrier function in the bone marrow. Lastly, a potential underlying mechanism of bone marrow vascular disruption was identified via increased angiogenic factors (MCP-1, IL-6 and VEGF) in the bone marrow and serum. These data suggest that bone marrow cells play critical roles in prostate cancer localization in the skeleton and warrant more intensive investigations of the impact of chemotherapeutic agents on bone marrow populations and tumor engraftment.

Bone Marrow Calcium Levels Correlate with Bone Marrow Hematopoietic Progenitor Cells

Osteoclast activity was recently found to stimulate mobilization of hematopoietic stem cells (HSCs). The calcium sensing receptor (CaSR) is essential for HSCs homing during the neonatal switch of hematopoiesis from liver to bone. This suggests an intriguing role of calcium in HSC regulation in bone. We hypothesized that the changes in the calcium concentration in bone affect hematopoietic progenitor cell populations. The purpose of this investigation was to determine if the bisphosphonate Zoledronic acid (ZA), alters calcium and impacts hematopoietic stem/progenitor cells (HPCs) and endothelial progenitor cells (EPCs) in the bone marrow microenvironment. Mice received ZA (5µg/mouse, twice/week) for 1, 3, 7, 14, 21 and 28d. At sacrifice, one hind limb was used for bone marrow calcium evaluation by flushing the whole bone marrow and performing a colorimetric assay. The other hind limb was used for bone marrow cell harvesting and HSC/EPC evaluation by flow cytometry. Histologic analysis of the vertebral bones and serum biochemistry were performed to confirm the effect of ZA. A long-term HSC reconstitution assay was performed with donor bone marrow cells from mice receiving ZA or vehicle for 28d. CD45.2 bone marrow cells were isolated from ZA-treated and vehicle-treated C57BL/6 mice and were mixed with equal numbers of CD45.1 cells from CD45.1 congenic mice. Cells were then injected intraorbitally into CD45.2/CD45.1 recipient mice that had been lethally irradiated. The frequency of the different cell populations was determined in the peripheral blood by flow cytometry using PE-CD45.1 and FITC-CD45.2 antibodies for 12 weeks after transplantation. Results showed: 1) ZA (versus vehicle) significantly decreased bone marrow calcium levels, serum TRAP5b and osteoclast numbers, increased bone area, and increased the percent of HPCs (Lin⁻, c-Kit⁺, Sca-1⁺, LSK cells) and EPCs (VEGFR2⁺VE-cadherin⁺ cells), but not HSCs (Lin⁻CD41⁻CD48⁺CD150⁺ cells) in the marrow, 2) bone marrow calcium levels negatively correlated with LSK cells in the bone marrow, and 3) ZA treated mice yielded more reconstituted T- and B-cells in the recipients at week 8 and 12 after bone marrow transplantation. This is the first report on bone marrow calcium levels in response to ZA and shed a light on the interaction of calcium and HPC/EPC populations in the bone marrow during anti-resorptive administration.